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Cartilage stress–relaxation is affected by both the charge concentration and valence of solution cations

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Summary

Objective: Understanding the mechanical functions of specific cartilage molecules such as aggrecan is important for understanding both healthy cartilage and disease progression. Cartilage is primarily composed of chondrocytes and an extracellular matrix consisting of multiple biopolymers, ions, and water. Aggrecan is one matrix biopolymer which consists of a core protein and multiple anionic glycosaminoglycans. Previous research has demonstrated that the stiffness of extracted aggrecan decreases under increased solution cation concentration, and the purpose of this study was to determine whether changes in solution ion concentration resulted in changes in tissue-level viscoelastic properties.

Methods: Middle-zone explants of bovine calf patellofemoral cartilage were harvested and cultured overnight before mechanical testing. Repeated stress–relaxation and cyclical loading tests were performed after equilibration in solutions of 0.15 M and 1 M NaCl and 0.075 M and 0.5 M $CaCl_2$. A stretched exponential model was fit to the stress–relaxation data. Storage and loss moduli were determined from the cyclical loading data.

Results: Changes in ionic strength and species affected both stress–relaxation and cyclical loading of cartilage. Stress–relaxation was faster under higher ionic strength. $CaCl_2$ concentration increases resulted in decreased peak stress, while NaCl increases resulted in decreased equilibrium stress. Storage and loss moduli were affected differently by NaCl and $CaCl_2$.

Conclusions: These results show that cartilage stress–relaxation proceeds faster under higher concentrations of solution cations, consistent with the theory of polymer dynamics. These data demonstrate the complexity of cartilage mechanical properties and suggest that aggrecan stiffness may be important in tissue-level cartilage viscoelastic properties.

Key words: Cartilage biomechanics, Flow-independent viscoelasticity, Polymer dynamics, Cartilage mechanics.

Introduction

Cartilage is the load-bearing surface that transmits compressive joint force during articulation. Aside from providing a low-friction surface for articulation, *in vivo* research has shown that articular cartilage deforms during articulation[1]. This deformation provides increased contact area which (1) decreases joint contact pressure and (2) provides stability during articulation. Because *in vivo* cartilage loading involves deformation, understanding the specific molecular contributions toward cartilage mechanical properties is important for understanding cartilage function.

Understanding the molecular origins of cartilage mechanical properties is also important for understanding degenerative cartilage diseases such as osteoarthritis. Animal models have shown that tissue-level mechanical behavior changes during osteoarthritis: in ACL transaction models, the complex shear and equilibrium aggregate moduli decrease with cartilage degeneration[2,3]. Understanding the molecular basis of the mechanical property degradation may provide therapeutic strategies and novel targets for osteoarthritis. This study used changes in solution ionic strength and species to investigate tissue-level cartilage viscoelastic properties.

The relative contributions of specific cartilage extracellular matrix molecules toward tissue-level mechanical properties are not sufficiently understood, and the purpose of this study is to investigate changes in cartilage viscoelastic properties caused by different ionic species and ionic strength solutions as a step toward understanding the role of aggrecan in cartilage material properties at the tissue-level. Recent studies have demonstrated that the stiffness and length of extracted aggrecan molecules decrease with increases in sodium ion concentration (Fig. 1)[4,5]. Additionally, intermolecular friction between aggrecan molecules increases with positive ion concentration[6]. However, questions remain as to how the solution ion concentration affects the tissue-level viscoelastic properties of cartilage.

A novel model for cartilage is based on the theory of polymer dynamics[7,8]. With respect to cartilage, polymer dynamics is defined as the motions and interactions of entangled polymers such as those of the cartilage extracellular matrix. The cartilage extracellular matrix is composed primarily of collagen and the proteoglycan aggregate[9,10], which are both polymers. As the cartilage extracellular matrix is

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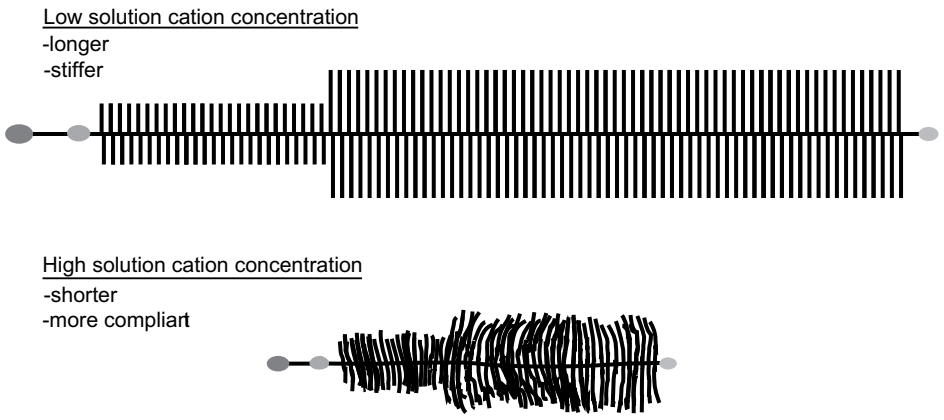

Fig. 1. Aggrecan conformation change under increased cation concentration. Top: aggrecan is stiff under low cation concentration. Bottom: aggrecan is more compliant with decreased length under high cation concentration. Previous research has shown that increased cation concentration results in decreases in aggrecan stiffness[4] and increases intermolecular aggrecan friction[6].

composed of polymers, polymer dynamics theory may be useful for studying the specific roles of cartilage matrix polymers in determining tissue-level mechanical properties.

Polymer dynamics theory predicts that stress–relaxation will proceed faster with shorter, more compliant (e.g., less stiff) molecules[11]: the greater mobility associated with increased compliance allows a faster equilibration to the applied deformation. Based on the known decrease in aggrecan stiffness with increased cation concentration and the polymer dynamics prediction of faster stress–relaxation with more compliant molecules, we hypothesized that (1) stress–relaxation would proceed faster and (2) tissue stiffness would decrease for cartilage samples under increased ion concentration. The objective of this study was to determine how changes in solution ion concentration affect tissue-level stress–relaxation and cyclical loading of articular cartilage.

Methods

Cartilage samples (diameter: 3.39 ± 0.01 mm) were aseptically harvested from bovine stifle joints and maintained in tissue culture for 4 or 5 days prior to mechanical testing. Explants were harvested from a predetermined location midway between the proximal and distal ends on the medial aspect of the patellofemoral groove and placed in Dulbecco's Modified Eagle's Medium (DMEM) containing antibiotics. The surface and deep zones were removed leaving middle-zone explants of a standard height (3.91 ± 0.03)[12], which were rinsed three times with DMEM and antibiotics and incubated in 12-well plates with 4 mL of chemically defined culture medium at 37°C in 5% CO_2. The chemically defined medium contained 0.1% bovine serum albumin (BSA), insulin, transferrin, selenium (ITS) (1 mg/mL, 0.55 mg/mL, and 0.67 mg/mL, respectively), 50 μg/mL L-ascorbic-acid-2-phosphate, 100 U/mL penicillin, and 100 μg/mL streptomycin. Medium was changed after 2 days in culture, and samples were tested after 5 days in culture.

To determine the effects of solution cation concentration on cartilage viscoelastic properties, samples were removed from culture medium, equilibrated in a low-strength ionic solution for 45 min, mechanically tested, equilibrated in a high-strength ionic solution for 45 min, and mechanically tested again. Low-strength ionic solutions were composed of 0.15 M NaCl and 0.075 M $CaCl_2$. High-strength ionic solutions were 1 M NaCl and 0.5 M $CaCl_2$. (Note that the solution strengths were chosen to provide equivalent charge concentrations between cations.) Eight samples were tested for each cation species. The length of equilibration was selected from a preliminary study where samples were equilibrated for 1 h in low ionic strength solutions, placed in the mechanical testing system under a small prestress, and then exposed to a high-strength ionic solution. The results showed that, at a 99% confidence level, the slope of the last minute of the stress–time curve was undetectable after 45 min, suggesting that concentration-gradient-driven diffusion of the high-strength ionic solution was complete.

Four groups of control samples were utilized to assess changes in mechanical properties during the experiment. The first group (NaLL, $n = 4$ samples) consisted of samples equilibrated in low-strength NaCl, mechanically tested, returned to low-strength NaCl for 45 min, and mechanically tested a second time. The second control group (NaHH, $n = 4$) consisted of samples equilibrated in high-strength NaCl, mechanically tested, returned to high-strength NaCl a second time for 45 min, and mechanically tested again. The third (CaLL, $n = 5$) and fourth (CaHH, $n = 6$) control groups consisted of similar treatments, but with low and high-strength $CaCl_2$ as the solutions.

To determine whether these changes in solution ion concentration affected the sample water content, separate samples ($n = 10$) from each group (NaLL, NaLH, NaHH, CaLL, CaLH, and CaHH) were blotted dry and weighed on a balance (±0.1 mg, Mettler-Toledo, AB-104s, Columbus, OH) after equilibration in each solution.

Viscoelastic mechanical testing consisted of unconfined compression stress–relaxation followed by cyclical loading using an Enduratec ELF 3200 uniaxial testing system. The sample diameter was measured before testing using digital calipers. Samples were placed between polished stainless steel loading platens in a heated testing chamber. The top platen was lowered at 50 μm/s until a 60 kPa prestress (actual prestress: 60.5 ± 0.125 kPa) was reached, and this prestress was allowed to relax for 4 min during which time the bath was filled with warmed solution and temperature control was initiated. The initial specimen height was defined as the height at which the preload was achieved. Samples were tested in the solutions which they were immersed in immediately prior to mechanical testing. For example, CaLH samples were tested first in 0.075 M $CaCl_2$ and second in 0.5 M $CaCl_2$, and CaHH samples were tested twice in 0.5 M $CaCl_2$.

Stress–relaxation tests were performed using 5% compression with a rapid platen displacement rate of 10 mm/s. Small-amplitude cyclical strains (~0.5%) were applied at frequencies of 0.01, 0.1, 1, 10, and 20 Hz after 300 s of relaxation. For stress–relaxation, data were collected at 180 Hz, and for cyclical loading sampling occurred at a minimum of 20 times the loading frequency. Apparent stresses were calculated by dividing the compressive force by the sample cross-sectional area measured immediately prior to the mechanical test.

Stress–relaxation parameters were determined by fitting a stretched exponential model[11,13,14] to the cartilage stress–relaxation data (Eq. (1)):

$$\sigma = \left(\sigma_{peak} - \sigma_{eq}\right) e^{-\left(\frac{t}{\tau}\right)^{\beta}} + \sigma_{eq} \tag{1}$$

σ_{peak} and σ_{eq} represent the peak and equilibrium stress which were defined by the experimental data. Note that the existence of σ_{eq} in the model implicitly models stress resulting from an elastic component of cartilage such as the cross-linked collagen. τ is the stress–relaxation time constant, related to the physical characteristics (e.g., temperature, polymer length, and concentration) of the system. Polymer dynamics theory predicts that τ will decrease upon decreases in polymer stiffness[11]. β is the stretching parameter, related to the specific type of polymer motion (e.g., Rouse or Reptation). Polymer theory predicts that β will decrease when the entangled polymer length increases[13]. This model represents stress–relaxation of polydisperse polymer systems such as cartilage[13,15]. τ and β were determined using nonlinear optimization to minimize the weighted square residuals between the model and the data. In addition to τ and β, a model-independent parameter, $\widehat{D}$, was used to quantify stress–relaxation [Eq. (2), Fig. 2(a)].

$$\widehat{D} = \int_{t=0}^{T} \frac{\sigma(t)}{\sigma_{peak}} dt - \frac{\sigma_{eq}}{\sigma_{peak}} T \tag{2}$$

Above, $t = 0$ references the time at which the peak stress was reached, T is the maximum time of the experiment (300 s for these experiments), and $\sigma(t)$ is the stress–relaxation function.

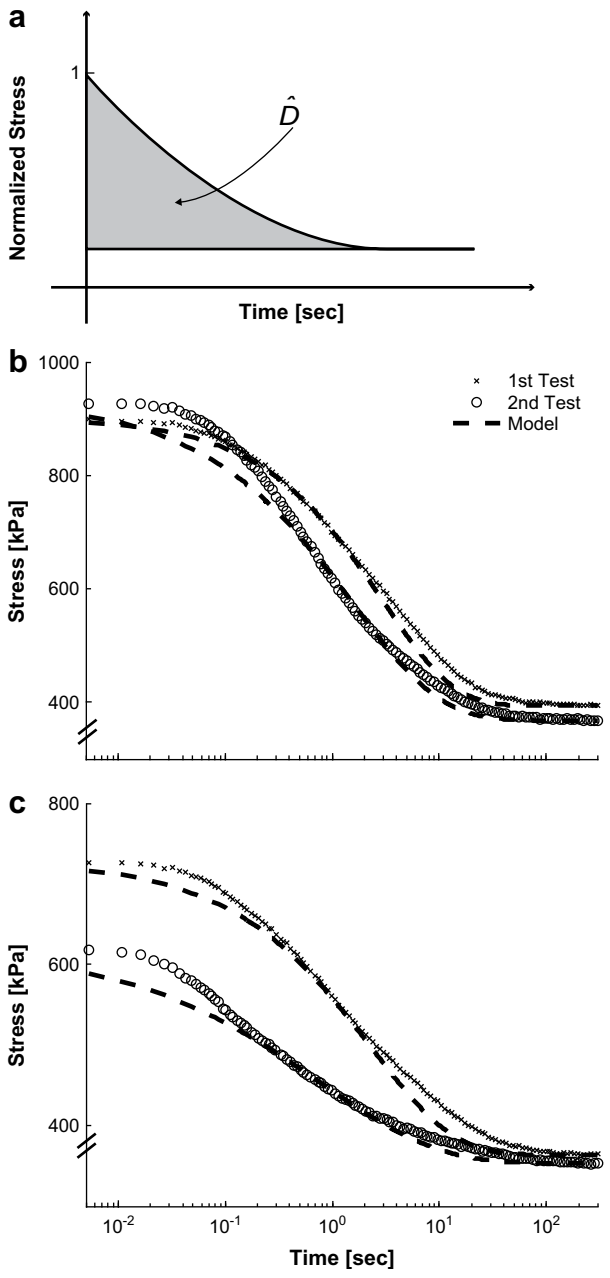

Fig. 2. Model-independent measure $\hat{D}$ and representative stress–relaxation data. (a) The value $\hat{D}$ is calculated as the total area (shaded region) between the normalized stress–relaxation data and the equilibrium stress, which is represented by the horizontal line in the figure. $\hat{D}$ is a model-independent measure of stress–relaxation analogous to a time constant. (b) first test in 0.15 M and second test in 1 M NaCl. Under higher NaCl concentration the peak stress increased and stress–relaxation proceeded faster. $R^2 = 0.94$ (first test) and 0.96 (second test). (c) first test in 0.075 M and second test in 0.5 M $CaCl_2$. Under higher $CaCl_2$ concentration, the peak stress decreased and stress–relaxation proceeded faster. $R^2 = 0.89$ (first test) and 0.84 (second test).

Cyclical loading experiments were analyzed by finding the phase lag of the stress compared to the strain. Storage and loss moduli were calculated as the in-phase and out-of-phase amplitude ratios of stress to strain, respectively. For frequencies greater than 0.1 Hz, we observed nonlinear cyclical behavior demonstrated by time-variant stress–responses during the first few loading cycles. In these cases, cyclical loading parameters were determined from the steady-state portion of the data.

Statistical analyses used paired t tests to determine the effects of repeated testing in the various experimental groups. For the cases where the repeated low-strength groups (LL) showed significant effects, the effects of changing ionic strength were compared with the low–low effects using Wilcoxon Rank-sum tests for non-parametric comparison of the median ratios of the first to the second value (e.g., comparison between the control (LL) and treatment (LH) groups using the ratios between the initial and final mechanical properties). The significance level was set *a priori* at 0.05. Quality of stretched exponential model fits was assessed using the coefficient of determination as described previously[16].

Results

Stress–relaxation proceeded faster at higher ionic concentration demonstrating that changes in solution ion concentration can change tissue-level cartilage viscoelastic properties and consistent with a role for aggrecan stiffness in cartilage stress–relaxation (Figs. 2(b), (c), and 3, Table II). The stretched exponential model fit the stress–relaxation data well ($R^2 = 0.92 \pm 0.01$). The dynamic stress–relaxation properties of $\hat{D}$, τ, and β decreased upon increase in cation concentration for both NaCl and $CaCl_2$ (groups NaLH and CaLH). Decreases in $\hat{D}$ and τ in the second test relative to the first test for the NaLH and CaLH groups demonstrate that stress–relaxation proceeded faster under higher ionic concentrations for both cations (both $P < 0.01$). $\hat{D}$ and τ were also smaller upon second testing for the CaHH controls ($P = 0.04$ and 0.02, respectively) but unchanged in the CaLL controls. The stretching parameter β was significantly smaller for the NaLH and CaLH groups ($P = 0.02$ and $P < 0.01$, respectively) and for the CaHH group ($P = 0.01$), but not for the CaLL group. The peak and equilibrium stress were affected differently by the different cation species: the peak stress decreased in the CaLH group ($P = 0.03$), while the equilibrium stress was decreased in the NaLH group ($P < 0.01$). Changes in NaCl concentration (the NaLH group) did not have significant effects on the peak stress and changes in $CaCl_2$ concentration (the CaLH group) did not have significant effects on the equilibrium stress. Specimen height decreased following equilibration in higher-strength ionic solutions (NaLH: $P = 0.03$, CaLH: $P = 0.02$, Table I). Small mass changes were observed in all calcium groups ($P \leq 0.04$, Fig. 6).

Cyclical loading results demonstrate that increases in bathing solution NaCl concentration result in smaller storage moduli at all measured frequencies, supporting the role of aggrecan stiffness in cartilage viscoelasticity (Fig. 4, Table III). Significantly smaller storage moduli were observed at all frequencies for the NaLH group (all $P < 0.01$). Significant differences were also observed in the NaLL group at frequencies greater than 0.1 Hz. However, non-parametric statistical analysis indicated that the NaLH decreases were greater than those observed in the NaLL control samples (all $P < 0.01$). No significant storage modulus differences were observed in any $CaCl_2$ groups.

The loss modulus was affected differently by sodium and calcium (Fig. 5, Table IV). NaLH samples exhibited loss modulus decreases at 0.01 and 0.1 Hz (both $P < 0.01$). At 0.01 Hz NaLL samples also exhibited smaller loss modulus values ($P = 0.03$), but non-parametric analysis revealed that the NaLH decreases were larger than the NaLL decreases ($P < 0.01$). Loss modulus decreases were observed in the CaLH group at frequencies of 0.1 Hz and higher (0.1 Hz: $P = 0.04$, 1–20 Hz: $P < 0.01$).

Discussion

The objective of this study was to determine whether changes in solution ion concentration and valence affected

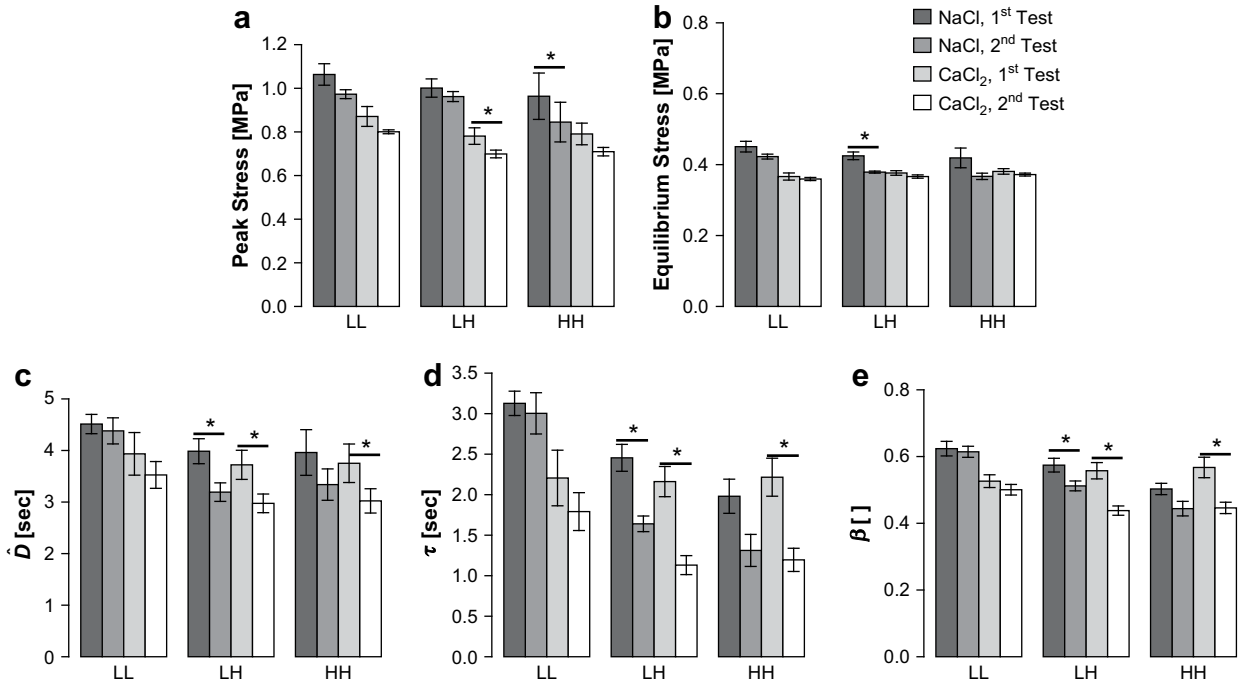

Fig. 3. Stress–relaxation results. (a) σ_{peak}, (b) σ_{eq}, (c) $\widehat{D}$, (d) τ, and (e) β. * indicates significant difference ($P < 0.05$) between first and second tests in a given group. Groups are LL (repeated low-concentration testing), LH (testing in low concentration followed by high) and HH (repeated high-concentration testing). The peak stress decreased upon testing in high-concentration $CaCl_2$ ($P = 0.03$). The equilibrium stress was significantly smaller upon testing in high-concentration NaCl ($P < 0.01$). Stress–relaxation proceeded faster under higher ionic concentrations for both NaCl and $CaCl_2$ as shown by smaller values of $\widehat{D}$ and τ ($P < 0.01$). $\widehat{D}$ and τ were also smaller for second tests in $CaCl_2$ ($P = 0.04$ and 0.02, respectively.) The stretching parameter β was significantly smaller for higher ionic concentrations of both species (NaCl, $P = 0.02$; $CaCl_2$, $P < 0.01$) and for second tests in 0.75 M $CaCl_2$ ($P = 0.01$).

tissue-level stress–relaxation and cyclical loading of articular cartilage. Previous research has demonstrated that aggrecan stiffness decreases with increases in NaCl concentration[4]. These data show that changes in bathing solution cationic strength and valence cause changes in cartilage viscoelastic properties, and in conjunction with previous studies[4] suggest that aggrecan stiffness is important in cartilage viscoelasticity.

Two limitations of this study must be considered: first, changes in ionic solution may affect multiple components of cartilage, so aggrecan-independent effects may be present in addition to effects mediated by aggrecan. For example, previous studies suggest that divalent calcium ions may create sacrificial bonds in bone[17,18] and that intra-fibrillar water content may vary with solution ion concentration[19,20]. This study is unable to differentiate between ionic-solution-induced and other mechanical property changes if multiple mechanisms exist (e.g., changes from both aggrecan stiffness and aggrecan-independent mechanisms such as sacrificial bonds within the collagen network or intra-fibrillar hydration). Importantly, collagen charge density is known to change with pH[21], but the solution pH was constant in these experiments. Second, changes in stress–relaxation due to relaxation-phase variations in the sample diameter between experimental groups may have contributed to the observed changes in stress–relaxation parameters. While this study did not measure the sample diameter during relaxation, previous research[22] has found that the specimen radius decreases by about 1.3% during stress–relaxation under 5% compression, whereas the presently observed changes in τ were 67% and 51% for the NaLH and CaLH groups, respectively.

Table I

*Height measurements prior to stress–relaxation tests. The specimen height was measured after a 60 kPa preload was applied at a displacement rate of 50 μm/s. After equilibration in a higher ionic strength solutions, the specimen height decreased (NaLH: *P = 0.03 and CaLH: #P = 0.02)*

Group	Specimen height [mm]	
	First test	Second test
NaLL	4.10 ± 0.15	4.12 ± 0.18
NaLH*	4.04 ± 0.11	3.98 ± 0.10
NaHH	4.03 ± 0.17	4.01 ± 0.18
CaLL	3.92 ± 0.08	3.91 ± 0.08
CaLH#	3.81 ± 0.11	3.75 ± 0.11
CaHH	3.81 ± 0.07	3.77 ± 0.05

Significant decreases in $\widehat{D}$, τ, and β were observed in the CaHH group [Fig. 3(c)–(e)]. However, no significant differences were observed in the CaLL group for these parameters. We tenatively conclude that the changes in $\widehat{D}$, τ, and β in the CaLH group result from the increase in divalent calcium ion concentration as opposed to repeated testing since the CaLH increases were significantly larger than the CaHH increases due to repeated testing.

Stress–relaxation proceeded faster at higher ionic concentrations than at lower ionic concentrations for both cation species as shown by the decreases in $\widehat{D}$ and τ (Fig. 3 and Table II). Previous research shows that increasing ionic strength results in both a decrease in aggrecan stiffness[4]

Table II
Stress–relaxation results

Group	Test	Peak stress [kPa]	Equilibrium stress [kPa]	$\widehat{D}$ [s]	τ [s]	β [–]
NaLL	First	1063 ± 49	451 ± 15	4.51 ± 0.19	3.13 ± 0.15	0.62 ± 0.02
	Second	973 ± 20	423 ± 7	4.38 ± 0.25	3.00 ± 0.25	0.61 ± 0.02
NaLH	First	1001 ± 42	425 ± 11	3.99 ± 0.24	2.45 ± 0.17	0.57 ± 0.02
	Second	962 ± 23	379 ± 3	3.19 ± 0.18	1.64 ± 0.10	0.51 ± 0.01
NaHH	First	964 ± 106	419 ± 28	3.96 ± 0.44	1.98 ± 0.21	0.50 ± 0.02
	Second	845 ± 91	367 ± 9	3.34 ± 0.30	1.31 ± 0.20	0.44 ± 0.02
CaLL	First	871 ± 46	366 ± 10	3.93 ± 0.41	2.21 ± 0.34	0.53 ± 0.02
	Second	801 ± 9	359 ± 4	3.53 ± 0.26	1.79 ± 0.23	0.50 ± 0.02
CaLH	First	781 ± 38	376 ± 6	3.72 ± 0.28	2.16 ± 0.19	0.56 ± 0.02
	Second	699 ± 18	366 ± 5	2.97 ± 0.18	1.13 ± 0.12	0.44 ± 0.01
CaHH	First	791 ± 50	381 ± 8	3.75 ± 0.37	2.22 ± 0.23	0.57 ± 0.03
	Second	709 ± 19	372 ± 4	3.02 ± 0.24	1.20 ± 0.14	0.45 ± 0.02

and an increase in intermolecular aggrecan friction[6]. The presently observed changes in $\widehat{D}$ and τ likely result from decreases in aggrecan stiffness, since increases in intermolecular friction would slow stress–relaxation[11]. Polymer dynamics theory predicts faster stress–relaxation with decreased molecular stiffness, and these data are consistent with polymer dynamics as a flow-independent mechanism of cartilage viscoelasticity. Polymer stress–relaxation proceeds faster for more compliant polymers because the increased molecular mobility associated with decreased stiffness allows faster equilibration to an applied deformation.

We observed decreases in β with increases in ion concentration for both cation species [Fig. 3(e)]. Theory predicts that changes in β may result from changes in the specific type of polymer motion, with decreased β values associated with increasing entanglement molecular weight[13]. The observed decreases in β with increased ion concentration are consistent with the hypothesis that aggrecan structural changes resulted in increased entanglement, which is possible considering the known decrease in aggrecan stiffness with increased ion concentration[4].

Divalent calcium and monovalent sodium had different effects on the peak and equilibrium stresses [Fig. 3(a) and

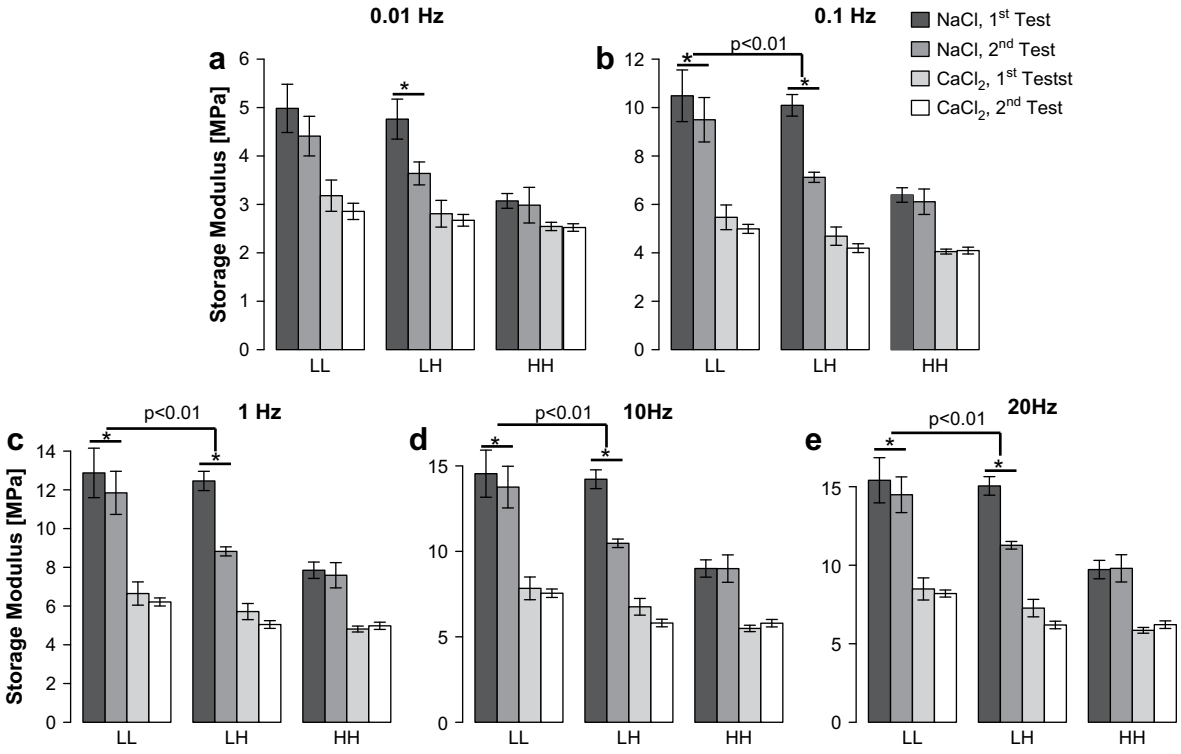

Fig. 4. Cyclical loading results, storage modulus. (a) 0.01 Hz, (b) 0.1 Hz, (c) 1 Hz, (d) 10 Hz, and (e) 20 Hz. Significantly smaller storage moduli were observed at all frequencies for the NaLH group (all $P < 0.01$). Significant differences were also observed in the NaLL group at frequencies greater than 0.1 Hz. Non-parametric statistical analysis indicated that the NaLH decreases were greater than those observed in the NaLL control samples (all $P < 0.01$). No significant storage modulus differences were observed in any $CaCl_2$ groups.

Table III
Storage modulus results

[MPa]		Loading frequency [Hz]				
Group	Test	0.01	0.1	1	10	20
NaLL	First	4.98 ± 0.50	10.49 ± 1.07	12.87 ± 1.28	14.55 ± 1.38	15.41 ± 1.44
	Second	4.41 ± 0.41	9.50 ± 0.92	11.84 ± 1.11	13.76 ± 1.22	14.49 ± 1.14
NaLH	First	4.76 ± 0.41	10.09 ± 0.45	12.46 ± 0.50	14.22 ± 0.55	15.05 ± 0.59
	Second	3.64 ± 0.24	7.12 ± 0.21	8.82 ± 0.23	10.47 ± 0.25	11.27 ± 0.24
NaHH	First	3.07 ± 0.15	6.39 ± 0.30	7.85 ± 0.42	9.00 ± 0.51	9.72 ± 0.59
	Second	2.98 ± 0.37	6.11 ± 0.53	7.59 ± 0.65	8.99 ± 0.80	9.80 ± 0.87
CaLL	First	3.18 ± 0.32	5.47 ± 0.51	6.65 ± 0.60	7.84 ± 0.66	8.49 ± 0.70
	Second	2.86 ± 0.17	4.99 ± 0.19	6.21 ± 0.21	7.55 ± 0.25	8.20 ± 0.22
CaLH	First	2.81 ± 0.28	4.69 ± 0.38	5.72 ± 0.42	6.76 ± 0.49	7.27 ± 0.56
	Second	2.67 ± 0.12	4.19 ± 0.18	5.05 ± 0.20	5.81 ± 0.49	6.19 ± 0.24
CaHH	First	2.54 ± 0.09	4.05 ± 0.10	4.81 ± 0.15	5.49 ± 0.19	5.85 ± 0.18
	Second	2.52 ± 0.08	4.09 ± 0.14	4.98 ± 0.19	5.80 ± 0.22	6.21 ± 0.24

(b)]. Increasing monovalent sodium concentration resulted in a decreased equilibrium stress and unchanged peak stress while increasing divalent calcium concentration resulted in decreased peak stress but unchanged equilibrium stress. The aggrecan-related portion of the equilibrium stress has been attributed to repulsive electrostatic interactions between glycosaminoglycan (GAG) chains[4,5]. Monovalent cations have longer Debye lengths than divalent cations, explaining why sodium better screens the equilibrium stress than does calcium for equal charge concentrations: the shorter Debye length associated with the low-concentration calcium may have completely screened the electrostatic component of the equilibrium stress, so no decrease in equilibrium stress was observed upon increased calcium concentration. Interestingly the peak stress effects were opposite to those of the equilibrium stress, with respect to cation species. That calcium increases resulted in decreased peak stress and loss modulus at frequencies of 0.1 Hz and greater (Figs. 3 and 5) suggests that the aggrecan structural change induced by

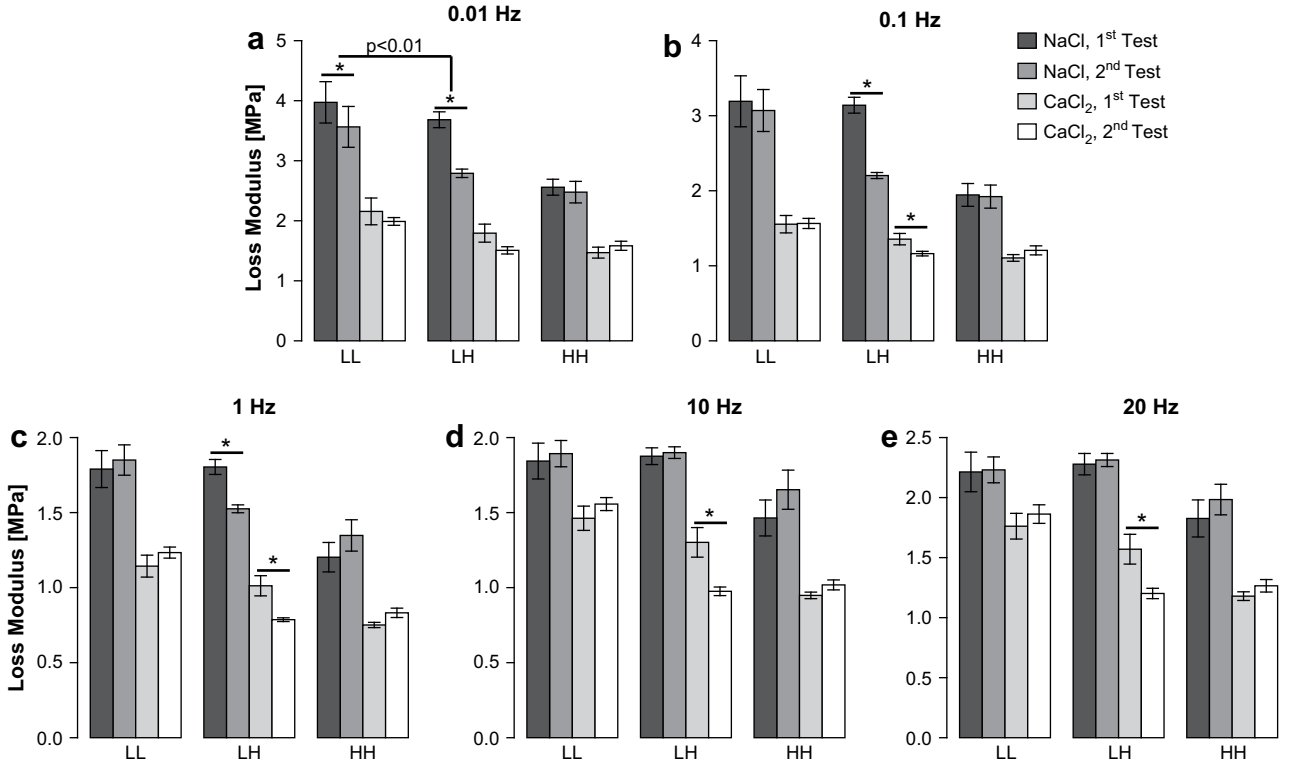

Fig. 5. Cyclical loading results, loss modulus. (a) 0.01 Hz, (b) 0.1 Hz, (c) 1 Hz, (d) 10 Hz, and (e) 20 Hz. NaLH samples exhibited loss modulus decreases at 0.01 Hz and 0.1 Hz (both $P < 0.01$). At 0.01 Hz NaLL samples also exhibited smaller loss modulus values ($P = 0.03$), but nonparametric analysis revealed that the NaLH decreases were greater than the NaLL decreases ($P < 0.01$). Loss modulus decreases were observed in the CaLH group at frequencies of 0.1 Hz and greater (0.1 Hz: $P = 0.04$, 1–20 Hz: $P < 0.01$).

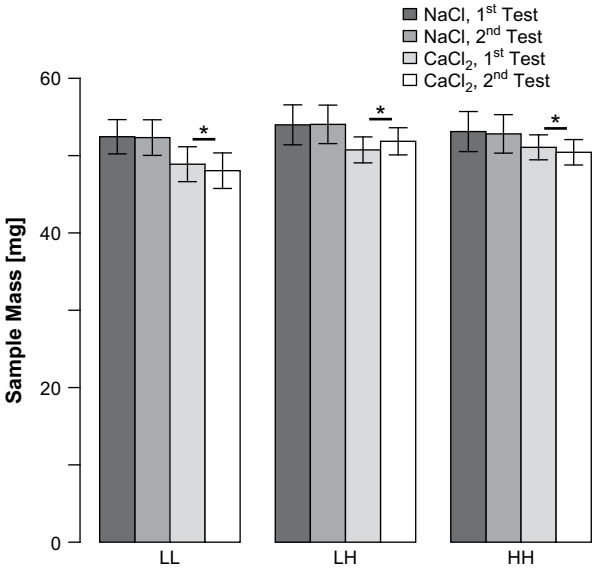

Fig. 6. Mass data as a measure of tissue hydration. No significant changes in tissue mass were observed in any of the sodium groups, but small changes were observed in all calcium groups (CaLL, $P < 0.01$; CaLH, $P < 0.01$, CaHH, $P = 0.04$). For the CaLH group, the relative mass change was $2.10 \pm 0.34\%$, but the relative τ change was $49.6 \pm 27.3\%$, suggesting that the changes in τ are independent of changes in water content.

divalent calcium results in a greater tissue-level dissipative effect than that which may be induced by monovalent sodium concentration increases. However, aggrecan-independent effects may also be involved, and further studies are required to evaluate these effects.

Monovalent sodium concentration increases resulted in significant decreases in the storage modulus at all testing frequencies (Fig. 4). At frequencies of 0.1 Hz and greater, the NaLL group also exhibited significant decreases in storage modulus upon repeat testing. However, the observed significant decreases in the NaLH group are supported both by their observed statistically significant difference relative to the NaLL group and by the absence of effects in the NaHH groups. The storage modulus is an in-phase measure of tissue stiffness, and the observed decreases in response to monovalent sodium increase are supportive of electrostatic interactions in tissue-level mechanical behavior because of the decrease in Debye length associated with increased ionic concentration[23]. Furthermore, the observation of decreased tissue stiffness in association with decreased aggrecan stiffness is consistent with polymer dynamics predictions[11], supporting polymer mechanisms in cartilage mechanical behavior.

The differential effects of sodium and calcium cations are evident in the loss modulus data (Fig. 5). Increasing the concentration of monovalent sodium resulted in decreases in the low-frequency (0.1 Hz and lower) loss modulus, while divalent calcium increases led to decreases at 0.1 Hz and higher. These data suggest that the aggrecan structural changes may vary with cation valence. In conjunction with the aforementioned peak stress changes, one interpretation is that the calcium-induced aggrecan conformation change results in increased load on the covalently cross-linked collagen network thus decreasing the dissipative contributions from the proteoglycan aggregate. However, additional experiments are required to confirm this.

Changing solution ion concentration may alter cartilage stress–relaxation due to interstitial fluid flow, which could confound the flow-independent interpretation presented herein. The time constant for stress–relaxation of a fluid-filled cylinder in unconfined compression[24] is determined by the hydraulic permeability, k, the tensile equilibrium aggregate modulus, H_{+a}, and the sample radius, r[25]:

$$\tau_{\text{Flow}} = \frac{r^2}{H_{+a}k} \tag{3}$$

Previous research suggests that k decreases upon increase in NaCl concentration for 18-month bovine specimens[26]. Using previously determined values of H_{+a}[25] and k[26], τ_{Flow} was estimated using the measured radii for the NaLH group (Table V). The values of $\widehat{D}$ and τ are substantially smaller than τ_{Flow}. Although it is impossible to exclude fluid flow, these estimates suggest that fluid flow occurs on a longer timescale than the dynamics observed in these experiments. In addition, the observation that the relative changes in τ and $\widehat{D}$ were much larger than the relative changes in tissue mass (a surrogate hydration measure) suggests that changes in stress–relaxation dynamics are not strongly dependent on tissue hydration and may result from the experimentally induced changes in cation concentration.

Table IV
Loss modulus results

[MPa]		Loading Frequency [Hz]				
Group	Test	0.01	0.1	1	10	20
NaLL	First	3.97 ± 0.34	3.19 ± 0.34	1.79 ± 0.12	1.84 ± 0.12	2.21 ± 0.16
	Second	3.56 ± 0.34	3.07 ± 0.28	1.85 ± 0.10	1.89 ± 0.09	2.23 ± 0.11
NaLH	First	3.68 ± 0.13	3.14 ± 0.11	1.80 ± 0.05	1.88 ± 0.06	2.28 ± 0.09
	Second	2.79 ± 0.07	2.20 ± 0.04	1.53 ± 0.03	1.90 ± 0.04	2.31 ± 0.09
NaHH	First	2.56 ± 0.13	1.94 ± 0.15	1.20 ± 0.10	1.46 ± 0.12	1.83 ± 0.15
	Second	2.48 ± 0.13	1.92 ± 0.15	1.35 ± 0.10	1.65 ± 0.13	1.98 ± 0.13
CaLL	First	2.16 ± 0.22	1.55 ± 0.12	1.14 ± 0.07	1.46 ± 0.08	1.76 ± 0.11
	Second	1.99 ± 0.06	1.56 ± 0.07	1.23 ± 0.04	1.56 ± 0.04	1.86 ± 0.08
CaLH	First	1.79 ± 0.15	1.36 ± 0.08	1.01 ± 0.01	1.30 ± 0.10	1.57 ± 0.12
	Second	1.51 ± 0.15	1.16 ± 0.03	0.79 ± 0.01	0.98 ± 0.03	1.20 ± 0.04
CaHH	First	1.47 ± 0.09	1.10 ± 0.04	0.75 ± 0.02	0.95 ± 0.02	1.18 ± 0.04
	Second	1.58 ± 0.08	1.21 ± 0.06	0.83 ± 0.03	1.02 ± 0.03	1.27 ± 0.05

Table V
The estimated time constants for fluid flow are smaller than the presently observed values of τ and $\widehat{D}$. τ_{Flow} was estimated based on hydraulic permeability values[30] of 3.03 and 2.38 $\times$ 10^{-15} $m^4(N\,s)^{-1}$ for 0.15 and 2 M NaCl, respectively and tensile aggregate modulus[25] of 13.2 MPa from Eq. (2). These estimates suggest that the effects observed in these experiments are independent of fluid flow

NaLH group	τ [s]	$\widehat{D}$ [s]	Flow τ [s]
0.15 M	2.45 ± 0.17	3.99 ± 0.24	71.0 ± 0.6
2 M	1.64 ± 0.10	3.19 ± 0.18	90.7 ± 0.3

This study demonstrated that changing the solution cation concentration and valence has effects on cartilage viscoelastic properties. Previous research demonstrates that aggrecan stiffness changes with solution cation concentration[4], and this study demonstrated that changes in solution cation concentration result in changes in tissue-level stress−relaxation properties. Together, these studies support the interpretation that aggrecan stiffness is important in tissue-level cartilage mechanics, consistent with the theory of polymer dynamics as a mechanism of flow-independent, or matrix, viscoelasticity. These data, in conjunction with previous studies, present a complex picture of cartilage mechanics that involves electrostatic interactions[4,27], fluid pressurization[28,29], polymer motion and interaction (present study), and potentially unknown mechanisms.

Conflict of interest

The authors have no financial interest in this research.

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References

1. Eckstein F, Lemberger B, Gratzke C, Hudelmaier M, Glaser C, Englmeier KH, *et al. In vivo* cartilage deformation after different types of activity and its dependence on physical training status. Ann Rheum Dis 2005;64(2):291−5.
2. Setton LA, Mow VC, Howell DS. Mechanical behavior of articular cartilage in shear is altered by transection of the anterior cruciate ligament. J Orthop Res 1995;13(4):473−82.
3. Sah RL, Yang AS, Chen AC, Hant JJ, Halili RB, Yoshioka M, *et al.* Physical properties of rabbit articular cartilage after transection of the anterior cruciate ligament. J Orthop Res 1997;15(2):197−203.
4. Dean D, Han L, Grodzinsky AJ, Ortiz C. Compressive nanomechanics of opposing aggrecan macromolecules. J Biomech 2006;39(14): 2555−65.
5. Dean D, Seog J, Ortiz C, Grodzinsky AJ. Molecular-level theoretical model for electrostatic interactions within polyelectrolyte brushes: applications to charged glycosaminoglycans. Langmuir 2003;19(13): 5526−39.
6. Han L, Dean D, Mao P, Ortiz C, Grodzinsky AJ. Nanoscale shear deformation mechanisms of opposing cartilage aggrecan macromolecules. Biophys J 2007;93(5):L23−5.
7. Fyhrie DP, Barone JR. Polymer dynamics as a mechanistic model for the flow-independent viscoelasticity of cartilage. J Biomech Eng 2003;125(5):578−84.
8. June RK, Barone JR, Fyhrie DP. Cartilage stress−relaxation described by polymer dynamics. In: Annual Meeting of the Orthopaedic Research Society. Chicago, IL, 2006.
9. Franzen A, Bjornsson S, Heinegard D. Cartilage proteoglycan aggregate formation. Role of link protein. Biochem J 1981;197(3):669−74.
10. Miller EJ, Matukas VJ. Chick cartilage collagen: a new type of alpha 1 chain not present in bone or skin of the species. Proc Natl Acad Sci U S A 1969;64(4):1264−8.
11. Doi M, Edwards SF. The Theory of Polymer Dynamics. Oxford, UK: Oxford University Press; 1988.
12. Khalafi A, de Gennes PG. Increased accumulation of superficial zone protein (SZP) in articular cartilage in response to bone morphogenetic protein-7 and growth factors. J Orthop Res 2007;25(3):293−303.
13. de Gennes P. Relaxation anomalies in linear polymer melts. Macromolecules 2002;35:3785−6.
14. June RK, Barone JR, Fyhrie DP. The temperature-dependence of cartilage stress−relaxation rate. In: Annual Meeting of the Orthopaedic Research Society. San Diego, CA; 2007.
15. Heinegard D. Polydispersity of cartilage proteoglycans. Structural variations with size and buoyant density of the molecules. J Biol Chem 1977;252(6):1980−9.
16. Huang CY, Soltz MA, Kopacz M, Mow VC, Ateshian GA. Experimental verification of the roles of intrinsic matrix viscoelasticity and tension−compression nonlinearity in the biphasic response of cartilage. J Biomech Eng 2003;125(1):84−93.
17. Fantner GE, Hassenkam T, Kindt JH, Weaver JC, Birkedal H, Pechenik L, *et al.* Sacrificial bonds and hidden length dissipate energy as mineralized fibrils separate during bone fracture. Nat Mater 2005; 4(8):612−6.
18. Yeni YN, Kim DG, Dong XN, Turner AS, Les CM, Fyhrie DP, *et al.* Do sacrificial bonds affect the viscoelastic and fracture properties of bone? Clin Orthop Relat Res 2006;443:101−8.
19. Basser PJ, Schneiderman R, Bank RA, Wachtel E, Maroudas A. Mechanical properties of the collagen network in human articular cartilage as measured by osmotic stress technique. Arch Biochem Biophys 1998;351(2):207−19.
20. Maroudas A, Bannon C. Measurement of swelling pressure in cartilage and comparison with the osmotic pressure of constituent proteoglycans. Biorheology 1981;18(3−6):619−32.
21. Frank EH, Grodzinsky AJ. Cartilage electromechanics. I. Electrokinetic transduction and the effects of electrolyte pH and ionic strength. J Biomech 1987;20(6):615−27.
22. Wong M, Ponticiello M, Kovanen V, Jurvelin JS. Volumetric changes of articular cartilage during stress relaxation in unconfined compression. J Biomech 2000;33(9):1049−54.
23. Buschmann MD, Grodzinsky AJ. A molecular model of proteoglycan-associated electrostatic forces in cartilage mechanics. J Biomech Eng 1995;117(2):179−92.
24. Armstrong CG, Lai WM, Mow VC. An analysis of the unconfined compression of articular cartilage. J Biomech Eng 1984;106(2):165−73.
25. Soltz MA, Ateshian GA. A conewise linear elasticity mixture model for the analysis of tension−compression nonlinearity in articular cartilage. J Biomech Eng 2000;122(6):576−86.
26. Lu XL, Sun DD, Guo XE, Chen FH, Lai WM, Mow VC. Indentation determined mechanoelectrochemical properties and fixed charge density of articular cartilage. Ann Biomed Eng 2004;32(3):370−9.
27. Frank EH, Grodzinsky AJ. Streaming potentials: a sensitive index of enzymatic degradation in articular cartilage. J Orthop Res 1987;5(4): 497−508.
28. Park S, Krishnan R, Nicoll SB, Ateshian GA. Cartilage interstitial fluid load support in unconfined compression. J Biomech 2003;36(12): 1785−96.
29. Grodzinsky AJ, Lipshitz H, Glimcher MJ. Electromechanical properties of articular cartilage during compression and stress relaxation. Nature 1978;275(5679):448−50.
30. Lux Lu X, Miller C, Chen FH, Edward Guo X, Mow VC. The generalized triphasic correspondence principle for simultaneous determination of the mechanical properties and proteoglycan content of articular cartilage by indentation. J Biomech 2007.